

A Comparative Study in Bone Decalcification Using Different Decalcifying Agents

Sudha Jimson, Balachander .N, K. M. K. Masthan, Rajesh Elumalai

Abstract: *Aim:* The aim of the study is to evaluate the efficacy of different decalcifying agent used to decalcify bone. Bone is the strongest part in the human. Two types of bones are seen cancellous bone and compact bone. Demonstration of bone is quite difficult than any other tissues. To obtain satisfactory paraffin or celloidin of bone inorganic calcium must be removed from the organic collagen matrix, cartilage and surrounding tissue. *Materials and methods:* All the samples collected were from the human mandible fixed in neutral buffered formalin for 24 hrs. Cut into pieces ranging from 2cm in size. The samples were decalcified using 10% formic acid, 5% nitric acid, neutral EDTA, HCL,5%,nitric + EDTA . *Results:* For 5% nitric acid the initial decalcification started on 9th day, for HCL on 14th day , formic acid on 25th day and 5%nitric acid +EDTA on 22nd day, neutral EDTA on 45th day.(graph1) The end point of decalcification for 5% nitric acid was 25days, HCl was 34days, 5 % nitric acid +EDTA was 46 days, 10%formic acid was 47days,for neutral EDTA was 61 days. *Conclusion:* According to our study among the comparison of the five decalcifying agents formic acid proves to be the best with moderate time for decalcification and with ribboning of sections, good nuclear staining and minimal edema.

Keywords: Decalcification, EDTA, End point test, Ammonium Oxalate.

1. Introduction

Bone is the strongest part in the human. Two types of bones are seen cancellous bone and compact bone. Demonstration of bone is quite difficult than any other tissues. To obtain satisfactory paraffin or celloidin of bone inorganic calcium must be removed from the organic collagen matrix, cartilage and surrounding tissue. This is called decalcification and is carried out by chemical agents either with calcium salts or with chelating agents that bind to calcium ions. Decalcifying bone is an ongoing challenging aspect of clinical, veterinary pathology and histology research laboratories⁶.The type of decalcifying agent used depends on the factors like urgency of case,degree of mineralization, extent of investigation, and staining techniques required [1]. Thus, Stevens *et al*[12] reported that the fixation process involves a series of chemical events that differ depending on the tissues to be preserved, and have as the main objective to avoid autolysis of the specimens.

Rapid decalcifiers effect staining. The reason for this is soft tissues are not exposed to acids as in the case of bone. In such cases the acidity of solution increases and the staining is affected in the cell nuclei which lead to the failure of nuclear chromatin to take up hematoxylin and other basic dyes. Decalcifying agents are classified into the following

Acid	Proprietary	Strong	Weak	Chelating
Decalcifiers	Decalcifiers	Inorganic A	Organic Acids	Agents
	a) RAPID eg:HCL b) SLOW- eg:Formalin/ Formic Acid	Nitric Acid, HCL	Formic Acid Acetic Acid Picric Acid	EDTA (ethylene diamine tetraacetic acid)

In our study we had compared the time taken ,quality of staining, sectioning difficulty, structural destruction in decalcifying a 2cm bone using a slow proprietary

decalcifier (10% formic acid) Rapid (HCL), strong inorganic acid (5% nitric acid), chelating agent (EDTA), and a combination of strong inorganic acid and chelating agent(5% nitric acid and neutral EDTA).

2. Materials and Methods

All the samples collected were from the human mandible fixed in neutral buffered formalin for 24 hrs. Cut into pieces ranging from 2cm in size. The samples were decalcified using 10% formic acid, 5% nitric acid, neutral EDTA, HCL,5%,nitric +EDTA .Each day all the 5 decalcifying solutions were chemically tested with ammonium oxalate for the procedure of calcium and the tissues were removed from the decalcifying agent rinse with running water and placed in afresh change of decalcifying solution. Bubble test was done to find the progress of decalcification. The end point of decalcification was tested by radiographs and comparing it with the radiograph before decalcification. (Fig 1)

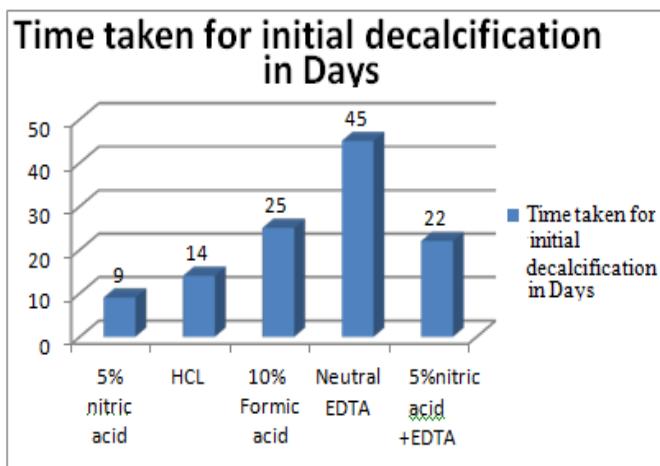
3. Results

For 5% nitric acid the initial decalcification started on 9th day, for HCL on 14th day , formic acid on 25thday and 5%nitric acid +EDTA on 22nd day, neutral EDTA on 45th day.(graph1) The end point of decalcification for 5% nitric acid was 25days, HCl was 34days, 5 % nitric acid +EDTA was 46 days, 10%formic acid was

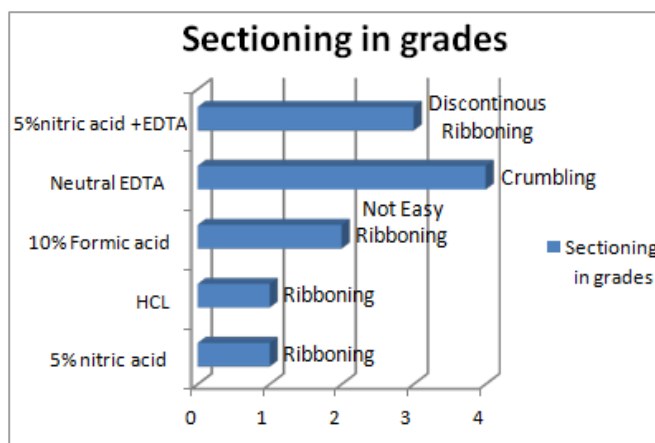
47days,for neutral EDTA was 61 days.(graph 2).Regarding the sectioning difficulties we graded I-ribboning,II-ribboning but not easy, III-discontinuous ribboning,IV-crumbling.5% nitric acid was graded as I,HCLwas graded as I, neutral EDTA was graded as IV,10% formic acid as II,5% nitric acid and EDTA as III.(graph3). Staining quality was poor for 5% nitric acid with high tissue swelling, and weakly stained nuclei where as 10% formic acid shows minimal tissue destruction and good staining of nuclei. EDTA, and EDTA+5% nitric acid stained moderately with minimum tissue destruction and good staining nuclei. HCl

had moderate swelling of tissue and weak nuclear staining. (graph 4&5) Fig: (2,3,&4).

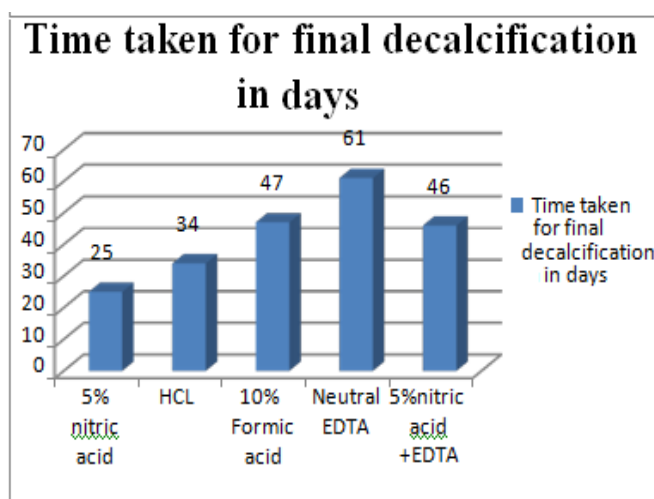
Type of decalcifying agent	Time taken for initial decalcification in Days	Time taken for final decalcification in days	Sectioning in grades	Swelling of tissue	Nuclear Staining
5% nitric acid	9	25	1(ribboning)	High	Weak
HCL	14	34	1(ribboning)	moderate	Weak
10% Formic Acid	25	47	2(ribboning but not easy)	minimal	Good
Neutral EDTA	45	61	4(crumbling)	moderate	Good
5%nitric acid+EDTA	22	46	3(discontinuous ribboning)	moderate	Good



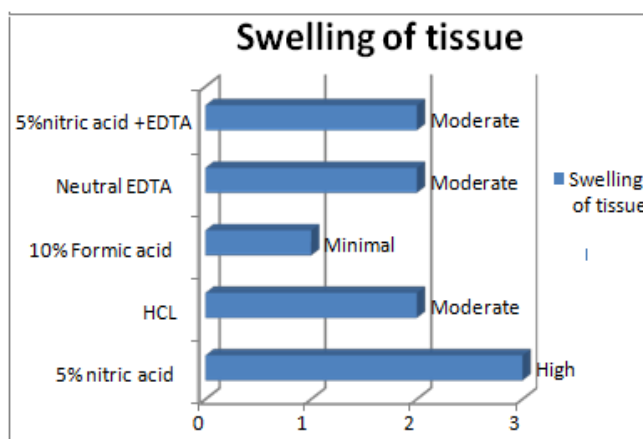
Graph 1



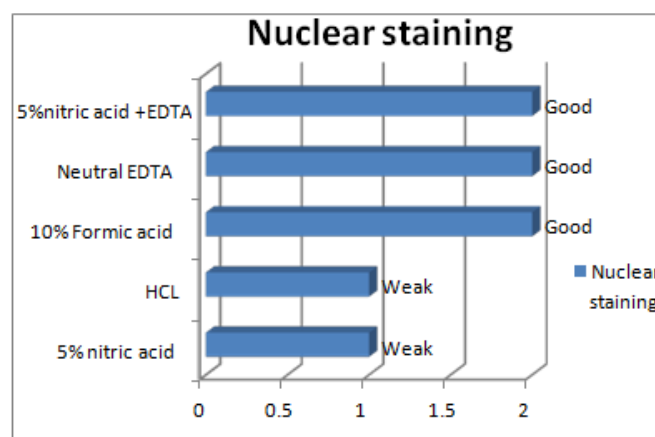
Graph 3



Graph 2



Graph 4



Graph 5

4. Discussion

Fixation time and the decalcifying agents used play a major role for the quality of histological section and accuracy of results. The use of decalcifying agents depends on four factors. Urgency of case, mineralization stage, aims of research and staining technique. Corca et al⁴ reported that the decalcification process causes important molecular and morphological alterations in the tissues such as edema, vacuolation, and ruptures not attributable to pathologic condition. In our study bone treated with 5% nitric acid decalcified quickly than other four. But there is edema and loss of nuclear staining. All acids effect tissue stability. These effects in 5% nitric acid is due to the fact that the time taken and the acidity of solution. Thus the quicker the decalcification the greater will be the injury and its effects in H&E sections [1, 12].

5% nitric acid is strong acid used in diagnostic urgency have a high free water ion concentration. They provoke significant alterations in soft tissues especially at cellular levels [11]. The decalcification process can be accelerated by agitation and increasing the temperature and medium.

Neutral EDTA is slower but the details are preserved and the nuclear staining is good. EDTA is a chelating agent slow in action and an excellent decalcifier for Immunohistochemistry and electron microscopy studies. In case of non urgency cases EDTA is the choice of decalcifying agent. Proteoglycans can be lost in certain fixatives and decalcifiers like EDTA, nitric and hydrochloric acids. This should be considered when choosing a decalcification method and agent to obtain optimal staining of cartilage for quantitative evaluation [6].

Acidic demineralizers cause distortion of the collagen fibers and deficiency in the affinity of histological stains for tissue structure which has been reported in connection with the use of EDTA [7]. On the contrary judged from the preservation of integrity of intercellular structures and stainability of tissue EDTA appears to surpass all other decalcifying agents. [8] Due to the advantage EDTA is used for research purposes and for electron microscopy [10]. EDTA is a tetradentate chelating agent and its neutral PH has the ability to remove calcium from bone in the form of calcium disodium edentate [5]. The disadvantage of EDTA is limited by the fact that it penetrates tissue poorly and works slowly. It is expensive electrolysis is slow and not suited for routine daily use. Neutral EDTA is used alone approx as 14% as neutral solution. Role of its decalcification depends on pH. Tissue sectioned with EDTA was the best to microtome knife. [14] Electrolytic decalcification is used with acid decalcifier which causes heat damage to specimen because of the application of current [3].

10% formic acid is in our study gave good results and it need not be watched carefully as other decalcifying agents. Mineral acids require prolonged periods to decalcify bone. 10% formic acid is the best all round decalcifier. Some commercial solutions combine formic acid with formalin to fix and decalcify tissue at the same time. 5% formic acid can preserve DNA for FISH and CGH studies [2].

The efficacy of decalcification of 10% formic acid is increased when buffered with citrate. Formic acid has the minimal shrinkage and loss of nuclear staining [9].

Extended decalcification time in HCl solutions may decrease nuclear staining. Hydrochloric acid is slower with greater distortion and edema than EDTA and weak nuclear staining which is so in our case. Acid decalcification hydrolysis DNA is not suitable for FISH and CGH. Microwave processing proved to be an efficient and reliable procedure for the decalcification of bones from laboratory animal species [13].

5. Conclusion

According to our study among the comparison of the five decalcifying agents formic acid proves to be the best with moderate time for decalcification and with ribboning of sections, good nuclear staining and minimal edema. Further study has to be conducted with combination of decalcifying agents and compare with other methods like sonication, microwave, and electrolytic. It is in any laboratories' best interest to examine the literature for the latest developments in the molecular biology, immunology, and histotechnology of decalcified bone before attempting a new procedure [6].

References

- [1] Bancroft Theory and practice of histological techniques, Chapter 14 Bone.
- [2] Brown R.S.J. of histochem, cyto chem 2002 Jn, 50(1):1113-5.
- [3] Caeson FL. Histo technology 2nd ed Chicago ASCP press 2007.
- [4] Corca CR, Oliveira MG, Carrard V, Lauxen IS, Oliveira OR, Barbieri BP. Avaliação das alterações microscópicas em material submetido a agentes descalcificadores. In: XV Salão de Iniciação Científica; Porto Alegre: ESEF; 2003. Res: 030. p. 554.
- [5] Goldstein et al (1974) Binding forms in the drug-receptor interaction. In: Goldstein A, Aronson L, Kalman SM (eds) Principles of drug action: the basis of pharmacology. Wiley & Sons, New York London, pp 11-12
- [6] Gayle Callis, Decalcification of Bone: Literature Review and Practical Study of Various decalcifying Agents. Methods. and Their Effects on Bone Histology, The Journal of Histotechnology, 1998, I Vol. 21, No. 1,
- [7] Honter HA, Nikiforuk G (1954) Staining reactions following demineralization of hard tissues by chelating and other decalcifying agents. J Dent Res 33:136-138
- [8] Janet Charman and Lynne Reid, The Effect of Decalcifying Fluids on the Staining of epithelial Mucins by Alcian Blue, Biotechnic & Histochemistry, 1972, Vol. 47, No. 4 : Pages 173-178
- [9] Karpagasekar sanjai JOMP 2012 May Aug (16(2)) 222-227
- [10] Martino CD, Floridi L, Marcante P. Morphological, histochemical and biochemical studies on grem cell mitochondria of normal rats. Cell Tissue Res. 1979; 196:1-22.
- [11] Marilene Issa Fernandes, Eduardo José Gaio, Cassiano Kuchenbecker Rosing, Rui Vicente Oppermann,, Pantelis Varvaki Rado, Microscopic qualitative

evaluation of fixation time and decalcification media in rat maxillary periodontium, Braz Oral Res,2007;21(2):134-9.

- [12] Stevens A, Lowe J, Bancroft JD. Bone. *In*: Bancroft JD, Theory and practice of histological techniques. 4th edition Pg309-39.
- [13] Shelley L. Gruntz; Vivian English, Expedited Bone Throughput Using Microwave decalcification, HISTOLOGIC,2011, Vol. XLIV, No. 1, 1-9.
- [14] Zappa et al Dent Med probl2005, 42:21-6. Comparison of different decalcification methods to hard teeth tissues morphological analysis. Dent Med Probl 2005;42:21-6